FEBS Letters 585 (2011) 2193–2198

FEBS Letters

journal homepage: www.FEBSLetters.org

Förster resonance energy transfer demonstrates a flavonoid metabolon in living plant cells that displays competitive interactions between enzymes

Kevin C. Crosby [a,b], Anna Pietraszewska-Bogiel [b], Theodorus W.J. Gadella Jr. [b], Brenda S.J. Winkel [a,*]

[a] The Department of Biological Sciences, Virginia Tech, Blacksburg, VA 24061, United States
[b] Swammerdam Institute for Life Sciences, Section of Molecular Cytology, Centre for Advanced Microscopy, University of Amsterdam, 1098 SM Amsterdam, The Netherlands

ARTICLE INFO

Article history:
Received 3 April 2011
Revised 26 May 2011
Accepted 29 May 2011
Available online 12 June 2011

Edited by Ulf-Ingo Flügge

Keywords:
Förster resonance energy transfer detected by fluorescent lifetime imaging microscopy
Flavonoid
Enzyme complex
Arabidopsis
Transient expression

ABSTRACT

We have used Förster resonance energy transfer detected by fluorescence lifetime imaging microscopy (FLIM-FRET) to provide the first evidence from living plants cells for the existence of a flavonoid metabolon. The distribution of flux within this system may be regulated by the direct competition of enzymes that catalyze key branch-point reactions, flavonol synthase 1 and dihydroflavonol 4-reductase, for association with the entry-point enzyme, chalcone synthase. Because the flavonoid enzymes were likely recruited from pathways of primary metabolism, our findings suggest a new general working model for the regulation of dynamic pathways in their native cellular context.

Structured summary of protein interactions:
CHS and **FLS1** physically interact by fluorescence resonance energy transfer (View interaction)
CHS and **DFR** physically interact by fluorescence resonance energy transfer (View Interaction 1, 2)
FLS1 physically interacts with **CHS** and **DFR** by competition binding (View interaction)

1. Introduction

The assembly of cooperating enzymes into multicatalytic complexes offers numerous advantages for cellular metabolism, including the potential to channel highly reactive or toxic intermediates between active sites and enhance the specificity and efficiency of biochemical pathways. Although controversial for many years, there are now numerous well-established examples of such enzyme assemblies [1–3].

We study the flavonoid biosynthetic pathway in Arabidopsis as a model enzyme complex. Flavonoids are specialized metabolites that are essential for plant growth, survival, and reproduction [4]. An early model for the intracellular organization of flavonoid metabolism had the phenylpropanoid and flavonoid enzymes forming a loosely-associated, linear array along the endoplasmic reticulum (ER) [5]. More recent experiments indicate that enzymes of the core flavonoid pathway associate via direct protein–protein interactions, including between those that catalyze non-consecutive reactions [6–8]. Specifically, yeast two-hybrid assays uncovered binary interactions between chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), and flavonol synthase 1 (FLS1).

Corroborating evidence for the associations among CHS, CHI, and F3H has come from affinity chromatography and co-immunoprecipitation experiments utilizing plant extracts, while immunofluorescence and immuno-electron microscopy showed that CHS and CHI colocalize in epidermal and cortex cells of the Arabidopsis root at the ER and, surprisingly, also in the nucleus [9]. Preliminary evidence from molecular modeling and surface plasmon resonance studies indicate that these enzymes may associate through relatively weak electrostatic interactions (Dana, Watkinson, Bowerman, and Winkel, unpublished data). Nevertheless, the precise nature of how these proteins interact to form multicatalytic complexes and regulate flux into competing branch pathways remains largely unknown, especially in the context of living cells.

Here we describe the use of Förster resonance energy transfer detected by fluorescence lifetime imaging microscopy (FLIM-FRET) to provide the first in vivo evidence for the existence of a flavonoid enzyme complex and support a model in which two key branchpoint enzymes, FLS1 and DFR, interact with CHS in a competitive manner. These findings suggest that a dynamic reorganization of loosely-organized enzyme complexes may provide a mechanism for rapidly redistributing pathway flux into endproducts with distinct physiological functions.

Abbreviations: ANS, anthocyanidin synthase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; FLS1, flavonol synthase 1; FLIM-FRET, Förster resonance energy transfer detected by fluorescence lifetime imaging microscopy

* Corresponding author. Address: 2125 Derring Hall (0406), Virginia Tech, Blacksburg, VA 24061, United States. Fax: +1 540 231 9307.
E-mail address: winkel@vt.edu (B.S.J. Winkel).

0014-5793/$36.00 © 2011 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2011.05.066

2. Materials and methods

2.1. Plasmid constructs

Using standard and Gateway (Invitrogen) cloning methods, cDNA sequences for CHS, FLS1, and DFR [10], fused to either syfp2, scfp3a [11], or mCherry [12] were inserted into the plant expression vector, p2∼gw7 [13], containing the enhanced cauliflower mosaic virus 35S promoter (see Supplementary material).

2.2. Preparation and transfection of Arabidopsis protoplasts

Arabidopsis mesophyll protoplasts were prepared from 3- to 4-week-old Columbia wild-type or *tt4* (Salk 020583) plants grown under short-day (11 h light) conditions and transfected as described in Yoo et al. [14]. Typically, 5–10 μg of donor plasmid and 10–20 μg of acceptor plasmid were used, a ratio that minimizes background from unbound donor. Competitor plasmids were transfected at the same concentration as acceptor plasmids. Protoplasts were incubated overnight for scfp3a/syfp2 pairs and for 36 h for syfp2/mCherry pairs prior to analysis.

2.3. Fluorescence lifetime imaging microscopy

Protoplasts were imaged in LabTek chambered coverglass (Nunc). Frequency-domain FLIM measurements were performed using the instrumental setup described in detail by van Munster and Gadella [15]. The objective used was a Zeiss plan Neofluar 40× 1.3 NA oil-immersion. Samples with a scfp3a donor were excited with either a 442 nm helium–cadmium laser (Melles-Griot) or a 440 nm diode laser (PicoQuant) modulated at 75.1 MHz; fluorescence was selected with a BP 460–500 nm emission filter. Samples with a syfp2 donor were excited using a 514 nm Argon laser (Melles-Griot) modulated at 75.1 MHz by an acousto-optic modulator; fluorescence was selected with a BP 530–560 emission filter. FLIM stacks of 12–36 phase steps were acquired in permutated recording order to reduce artifacts due to photobleaching [16]. Software for acquisition, processing, and analysis of the data was written in MATLAB 6.1 (Mathworks). Figures were generated using an ImageJ macro. Data were tabulated and graphs and tables were prepared in Excel (Microsoft). FRET efficiencies were calculated using the formula $E = 1 - (\tau_{DA}/\tau_{D})$. *P* values were calculated in Microsoft Excel using a two-tail Student's *t*-test with Welch's correction.

3. Results

3.1. CHS, FLS1, and DFR exhibit direct interactions in Arabidopsis protoplasts

Three enzymes of the core flavonoid biosynthetic pathway were selected for this study; CHS catalyzes the first committed step in flavonoid biosynthesis, while FLS1 and DFR lie at a major branch point in the pathway and share common substrates, the dihydroflavonols (Fig. 1). A series of fluorescent protein fusion constructs under control of the e35S promoter to allow for transient expression in Arabidopsis mesophyll protoplasts were made for each enzyme utilizing the monomeric cyan and yellow variants, SCFP3A and SYFP2 [11], and the monomeric red fluorescent protein, mCherry [17]. Immunoblot analysis using an anti-GFP antibody showed that full length fusion proteins were produced in protoplasts transfected with these construct (not shown).

FLIM analysis showed a decrease in lifetime of FLS1-scfp3a when this protein was expressed together with either syfp2-CHS or CHS-syfp2 (Fig. 2). Although there was a difference in mean reductions across experiments depending upon the orientation of CHS vis-à-vis syfp2 (Fig. 2; Table 1), these were minor. Substantially smaller decreases in lifetime were observed for scfp3a-FLS1 in the presence of either CHS acceptor construct, suggesting that the presence of the fluorescent protein at the FLS1 N-terminus may interfere with its interaction with CHS. The co-expression of scfp3a-DFR with syfp2-CHS or CHS-syfp2 also showed a shift to shorter lifetimes from those seen for scfp3a-DFR alone (Table 1). Once again, the orientation of the fluorescent protein with regard to CHS had little impact on the relative FRET levels. This provides strong evidence for the interaction of CHS with both FLS1 and DFR in vivo. Previous yeast two-hybrid assays have suggested that FLS1 and DFR may also interact with each other [7]. Indeed, FLIM-FRET measurements corroborate these earlier results because the scfp3a fluorescence lifetime of scfp3a-DFR is reduced by 8% or 9% in the presence of FLS1-syfp2 or syfp2-FLS1, respectively (Table 1).

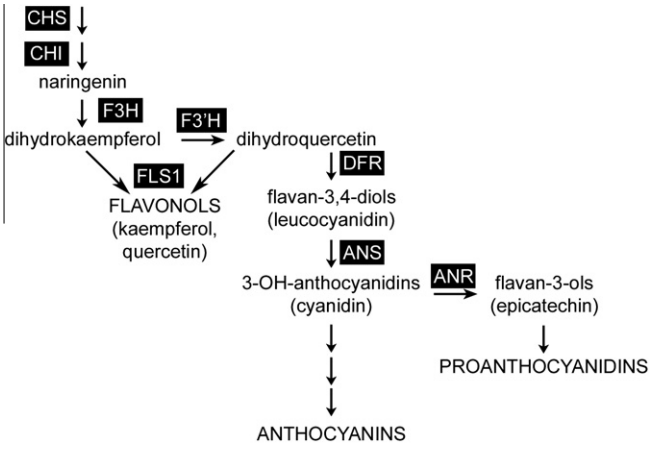

Fig. 1. Schematic of the core flavonoid pathway in Arabidopsis. Enzyme names are shown in black boxes and the three major classes of flavonoid endproducts are indicated in capitals. *Abbreviations:* CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase, FLS1, flavonol synthase 1; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase.

FRET efficiencies (*E*) based on $\tau(\varphi)$ were calculated for the FLS1/CHS, the DFR/CHS, and the DFR/FLS1 pairs (Table 1). These *E* values (≈10%) are comparable to what has been observed in studies of other protein pairs in plant systems using time-resolved based methods [18,19]. By way of comparison, a linked syfp and scfp3a construct showed FRET efficiencies of 26.5% [11]. For both the FLS1/CHS and the DFR/CHS pairs, the shifts observed in $\tau(m)$ were much less pronounced than those in $\tau(\varphi)$. This could be attributed to the presence of multiple populations of donor molecules exhibiting different levels of FRET; when there is a heterogeneous population of donor molecules, quenched (FRETing) and non-quenched (non-FRETing), the non-FRETing donors will contribute disproportionately to $\tau(m)$ measurements, resulting in a trend that can be represented by $E(\varphi) > E(m)$ [20].

3.2. FLS1 disrupts the CHS–DFR interaction

Having established that both FLS1 and DFR interact with CHS, we next sought to examine if CHS could bind to each of these proteins simultaneously. For these experiments, syfp2-DFR and mCherry-CHS were used as the donor/acceptor pair, while FLS1-scfp3a was co-expressed as a competitor. The scfp3a tag on FLS1 allowed us to verify expression of the competitor, but did not interfere spectroscopically with energy transfer from syfp2 to mCherry (Table 2). A decrease in donor lifetime was observed when expressing the syfp2-DFR/mCherry-CHS pair that was comparable to that seen earlier for scfp3a-DFR/syfp2-CHS. Notably, when FLS1-scfp3 was expressed at the same time, the donor lifetime for syfp3a-DFR shifted to a value close to that of the donor alone (Fig. 3; Ta-

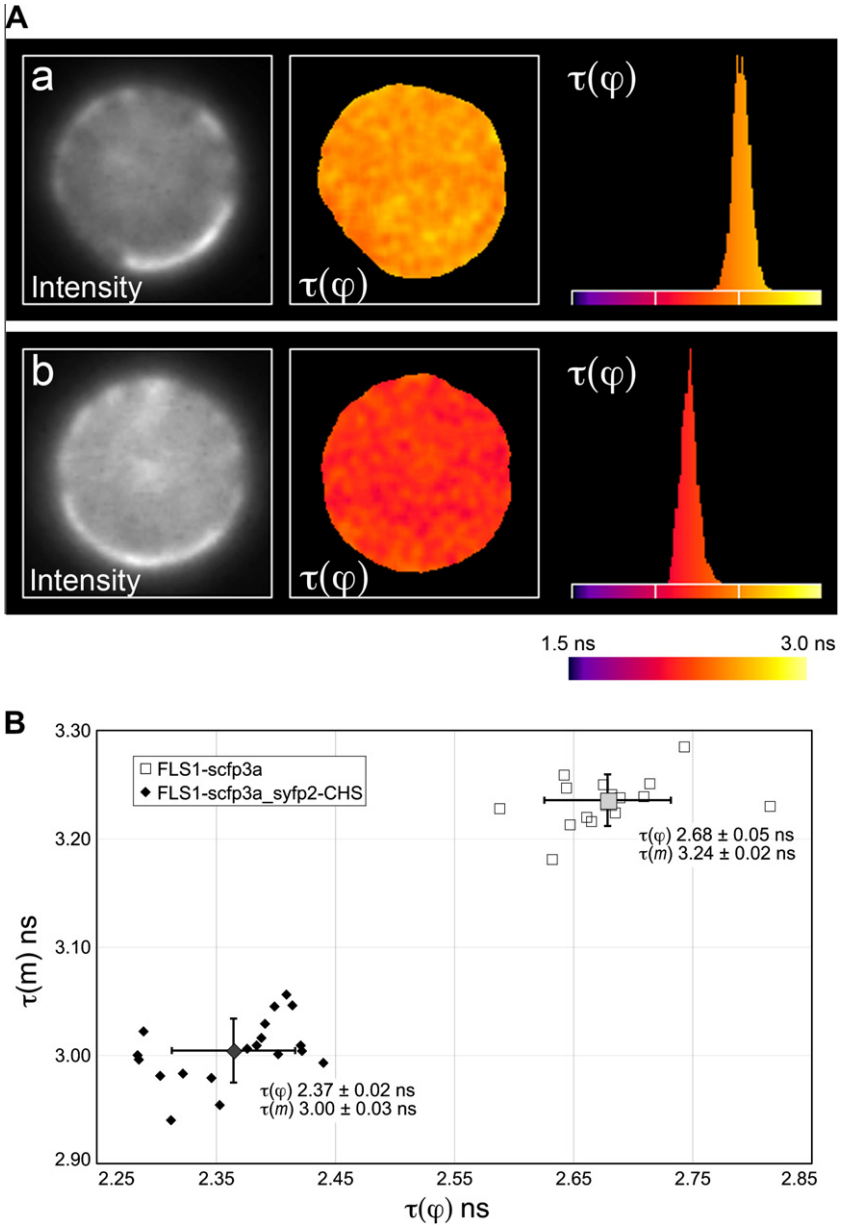

Fig. 2. Interaction of FLS1 and CHS as shown by FLIM-FRET analysis. (A) Images from representative protoplasts showing intensity, phase lifetime map-$\tau(\varphi)$, and 1-D lifetime histogram for FLS1-scfp3a. Top panels (a) are images from a protoplast expressing FLS1-scfp3a alone and bottom panels (b) are from a protoplast expressing both FLS1-scf3a and syfp2-CHS. (B) Scatterplot showing distribution of phase lifetimes, $\tau(\varphi)$, plotted against modulation lifetimes, $\tau(m)$, for protoplasts expressing FLS1-scfp3a (smaller open squares) and FLS1-scfp3a + syfp2-CHS (smaller closed diamonds). Mean lifetimes (larger square and diamond) and standard deviation for each sample set are also shown.

ble 2), while unfused scfp3a did not affect syfp2-DFR to mCherry-CHS FRET. Additionally, the scfp3a-FLS1 construct, which showed lower FRET efficiencies with syfp2-CHS and CHS-syfp2 alone (Table 1), exhibited only minimal competition.

The observed interference of the CHS–DFR association by FLS1 could occur through two possible mechanisms: the two enzymes could compete for a common or overlapping interaction site on CHS; alternatively, the ability of DFR to bind CHS could be impaired by the formation of FLS1–DFR complexes. Although each of these scenarios is possible and they are not necessary mutually exclusive, we favor the former. Our FLIM-FRET results show that attaching a fluorescent protein to the N-terminus of FLS1 seems to compromise its ability to bind with CHS. This same FLS1 construct also fails to appreciably disrupt the interaction between DFR and CHS. Yet, it does not appear to have an impact on the association with DFR, which occurs equally well whether the fluorescent protein is located at the N- or C-terminus of FLS1. If the mechanism by which FLS1 interfered with the DFR/CHS association was through a sequestering of DFR away from CHS, then both constructs should abrogate the FRET levels of the DFR–CHS pair equivalently. Therefore we believe our data are most consistent with a model where the disruption of the DFR–CHS interaction is dependent upon FLS1 binding to CHS, resulting in reorganization of the flavonoid complex and redistribution of flux into flavonol biosynthesis.

4. Discussion

The endproducts that are produced by the flavonoid pathway at different times and locations within the plant appear to be controlled by a number of different mechanisms, which may allow the cell to fine tune its response to developmental and

Table 1
Averaged data from representative FLIM-FRET experiments.

Donor	Acceptor	n[a]	Mean phase lifetime [$\tau(\varphi)$][b]	Modulation lifetime [$\tau(m)$][b]	Difference in mean donor lifetimes [$\Delta\tau(\varphi)$][c]	FRET efficiency [$E\tau(\varphi)$][d] (%)
FLS1-scfp3a	–	15	2.68 ± 0.05	3.24 ± 0.02	–	
	syfp2-CHS	19	2.37 ± 0.02	3.00 ± 0.03	0.31**	11.7
	CHS-syfp2	19	2.40 ± 0.06	3.00 ± 0.04	0.28**	10.4
FLS1-scfp3a	–	17	2.37 ± 0.09	3.18 ± 0.06	–	
	syfp2	17	2.39 ± 0.09	3.18 ± 0.10	−0.02	−0.7
scfp3a-FLS1	–	13	2.38 ± 0.05	3.02 ± 0.03	–	
	syfp2-CHS	5	2.22 ± 0.08	2.96 ± 0.04	0.16*	6.6
	CHS-syfp2	13	2.26 ± 0.07	2.95 ± 0.03	0.11**	4.8
scfp3a-DFR	–	11	2.42 ± 0.14	3.04 ± 0.06	–	
	syfp2-CHS	13	2.18 ± 0.14	2.90 ± 0.02	0.24**	10.0
	CHS-syfp2	12	2.14 ± 0.15	2.88 ± 0.13	0.28**	11.5
scfp3a-DFR	–	24	2.37 ± 0.12	3.03 ± 0.08	–	
	syfp2-FLS1	16	2.15 ± 0.13	2.95 ± 0.08	0.19**	9.0
	FLS1-syfp2	16	2.18 ± 0.11	2.89 ± 0.06	0.21**	8.0
scfp3a-DFR	–	17	2.29 ± 0.08	3.12 ± 0.03	–	
	syfp2	17	2.26 ± 0.06	3.08 ± 0.06	0.03	1.4

[a] Number of protoplasts measured per experiment.
[b] Values are given in nanoseconds ± standard deviation.
[c] Between donor lifetimes in the absence and presence of acceptor.
[d] Based on the mean phase lifetimes.
* Indicates a p-value of <0.05.
** p-value <0.001 as determined by a Student's t-test.

Table 2
Averaged data from representative FLIM-FRET competition experiments.

Donor	Acceptor	Competitor	n[a]	Mean phase lifetime [$\tau(\varphi)$][b]	Modulation lifetime [$\tau(m)$][b]	Difference in mean donor lifetimes [$\Delta\tau(\varphi)$][c]	FRET efficiency [$E\tau(\varphi)$][d] (%)
syfp2-DFR	–	–	16	2.77 ± 0.10	3.19 ± 0.03	–	
	mCherry-CHS	–	16	2.44 ± 0.16	3.06 ± 0.06	0.33**	11.8
	mCherry-CHS	FLS1-scfp3a	16	2.68 ± 0.10	3.11 ± 0.06	0.09††	3.2
syfp2-DFR	mCherry-CHS	scfp3a-FLS1	16	2.54 ± 0.17	3.07 ± 0.04	0.23	8.5
syfp2-DFR	mCherry-CHS	scfp3a	15	2.44 ± 0.13	3.02 ± 0.05	0.33	11.8

[a] Number of protoplasts measured per experiment
[b] Values are given in nanoseconds ± standard deviation
[c] Between donor lifetimes in the absence and presence of acceptor
[d] Based on the mean phase lifetimes
** p-value < 0.001 as determined by a Student's t-test.
†† p-value < 0.001 for the difference in the mean lifetimes for the donor/acceptor/competitor set versus mean lifetime for donor/acceptor set.

environmental stimuli. Regulation at the level of transcription is one important factor, as the expression of DFR and other enzymes involved in the synthesis of anthocyanins and proanthocyanidins is distinct from CHS, CHI, FLS1, and other genes early in the pathway [21,22]. There is also evidence for post-transcriptional mechanisms, including negative regulation of the expression and catalytic activity of DFR by flavonols, the product of FLS1 [23,24].

Our findings suggest that competitive enzyme interactions provide another means for distributing flux into the different branches of the flavonoid pathway. FLS1 and DFR were both shown to interact with CHS in living protoplasts, which corroborates our previous in vitro data for associations between these enzymes [6–8]. Because these protein interactions involve non-consecutive enzymes, our results provide further evidence for the existence of a globular superstructure with CHS functioning as a hub. This is an emerging theme for multienzyme systems, with other recent examples coming from the eukaryotic fatty acid synthase and yeast sterol biosynthetic complexes [25,26]. Moreover, FLS1 and DFR appear to bind to CHS in a mutually-exclusive manner. The structural basis for this interference, whether due to competition for common or overlapping binding sites, an induced change in the conformation of CHS, or some other mechanism, remains to be determined. It also remains possible that DFR may be sequestered away from CHS through its association with FLS1, although this seems less likely since fusion of the fluorescent tag to the N-terminus of FLS1 appears to impact its ability to interfere with the CHS–DFR interaction and also impedes the association of FLS1 with CHS but not with DFR. Nevertheless, the functional implications for regulating flux into branch pathways that utilize common substrates are clear. As DFR and FLS1 lie at a point of divarication between flavonols and the anthocyanins, it is easy to envision one mode of modulating flux between these branches is by the association and dissociation of the relevant biosynthetic enzymes with a core complex.

There is already strong genetic evidence that flavonoid enzymes use metabolic channeling to distribute flux between branch pathways in intact plant cells. For example, FLS1 knockout lines accumulate anthocyanins at levels nearly double those of wild-type plants, indicating that precursors normally channeled to flavonols are instead used for anthocyanin synthesis [7,23]. This is surprising given that the related flavonoid enzyme, anthocyanidin synthase (ANS), exhibits high FLS activity in vitro [27], thus indicating that ANS cannot access dihydroflavonols in vivo, even in the absence of FLS1. One explanation is that ANS only associates with other flavonoid enzymes when DFR is present and channeling anthocyanidins, the primary in vivo substrate for ANS, directly to this enzyme.

A mechanism for regulating metabolic flux via enzyme interactions has potential importance well beyond flavonoid biosynthesis, especially since this pathway is composed of members of enzyme superfamilies such as polyketide synthases, 2-oxoglutarate dependent dioxygenases, and cytochrome P450 hydroxylases that are

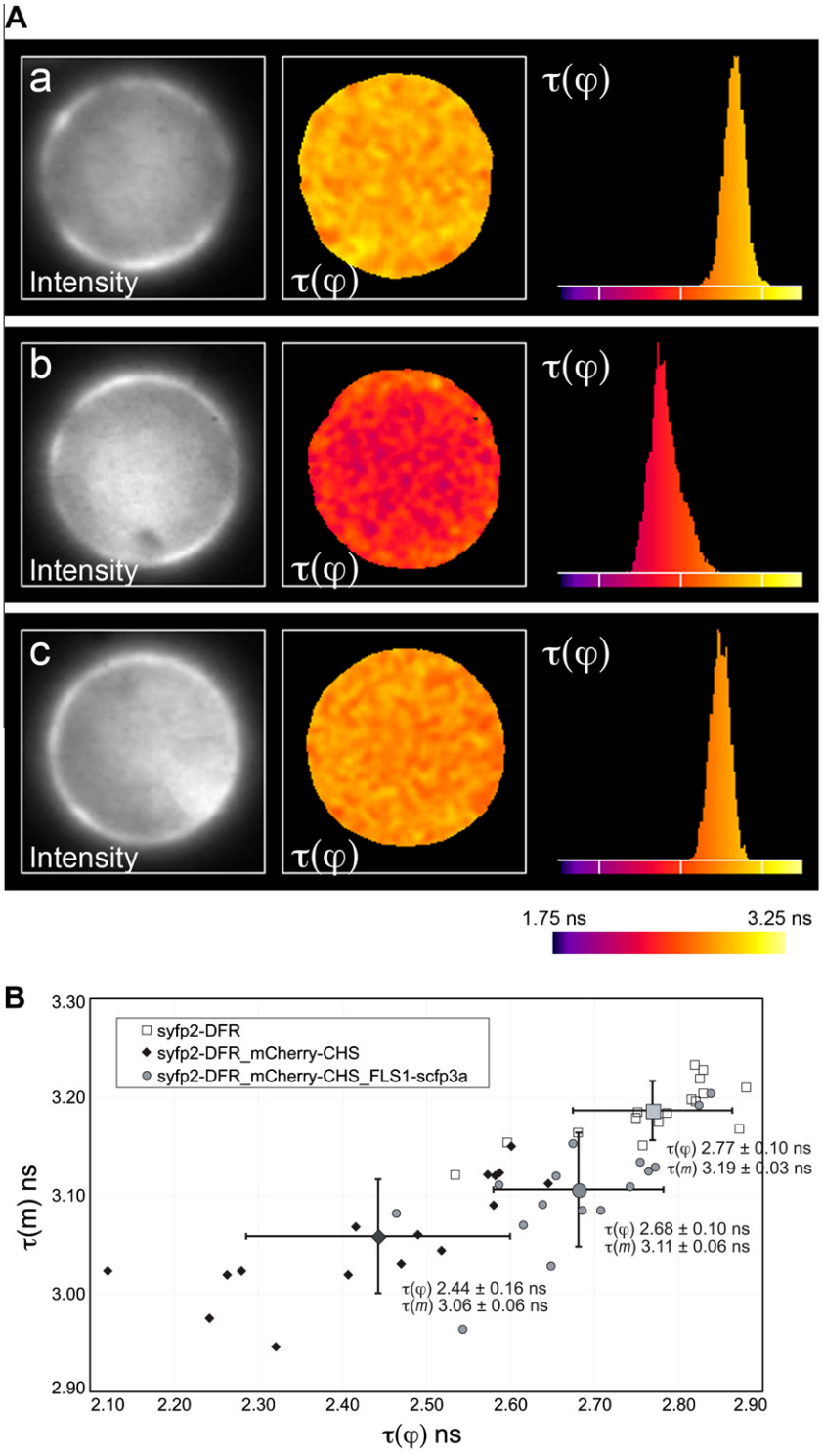

Fig. 3. FLS1 interferes with DFR–CHS interaction. (A) Images for representative protoplasts showing intensity and lifetime data for syfp2-DFR. Top panels (a) syfp2-DFR; middle panels (b) syfp2-DFR + mCherry-CHS; bottom panels (c) syfp2-DFR + mCherry-CHS + FLS1-scfp3a. (B) Scatterplot showing distribution of phase lifetimes, $\tau(\varphi)$, plotted against modulation lifetimes, $\tau(m)$, for protoplasts expressing syfp2-DFR (smaller open squares); syfp2-DFR + mCherry-CHS (smaller closed diamonds); syfp2-DFR + mCherry-CHS + FLS1-scfp3a (smaller shaded circles). Mean lifetimes (larger square, diamond, and circle) and standard deviation for each sample set are also shown.

fundamental features of many other pathways [28,29]. Moreover, a growing number of pathways appear to be assembled around a dimeric enzyme that, like CHS, may serve as a central organizing "hub" (e.g. [30]). If multi-enzyme complexes are as pervasive as many believe [31–33], then the regulation of metabolic channeling via dynamic and variable protein interactions may well be a common feature of cellular metabolism.

Acknowledgements

We thank Roger Tsien for the kind gift of the mCherry clone. We are also grateful to Joachim Goedhart, Laura van Weeren, and Merel Adjobo-Hermans for technical assistance and many stimulating discussions. This work was supported by grants from the NSF Molecular Biochemistry (MCB-0445878) and IGERT

(DGE-0523658) programs, the EU-FP6 Marie Curie Training Network Nodperception program (MRTN-CT-2006-035546), and the Virginia Tech Graduate Research and Development Program.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.05.066.

References

[1] Conrado, R.J., Varner, J.D. and DeLisa, M.P. (2008) Engineering the spatial organization of metabolic enzymes: mimicking nature's synergy. Curr. Opin. Biotechnol. 19, 492–499.
[2] Srere, P.A. and Ovadi, J. (1990) Enzyme–enzyme interactions and their metabolic role. FEBS Lett. 268, 360–364.
[3] Winkel, B.S.J. (2009) Metabolite channeling and multi-enzyme complexes in: Plant-derived Natural Products – Synthesis, Function and Application (Osborne, A.E. and Lanzotti, V., Eds.), pp. 195–208, Springer, New York.
[4] Winkel-Shirley, B. (2001) Flavonoid biosynthesis: a colorful model for genetics, biochemistry, cell biology and biotechnology. Plant Physiol. 126, 485–493.
[5] Hrazdina, G. and Jensen, R.A. (1992) Spatial organization of enzymes in plant metabolic pathways. Annu. Rev. Plant Physiol. Plant Mol. Biol. 43, 241–267.
[6] Burbulis, I.E. and Winkel-Shirley, B. (1999) Interactions among enzymes of the *Arabidopsis* flavonoid biosynthetic pathway. Proc. Natl. Acad. Sci. USA 96, 12929–12934.
[7] Owens, D.K., Alerding, A.B., Crosby, K.C., Bandara, A.B., Westwood, J.H. and Winkel, B.S.J. (2008) Functional analysis of a predicted flavonol synthase gene family in Arabidopsis. Plant Physiol. 147, 1046–1061.
[8] Owens, D.K., Crosby, K.C., Runac, J., Howard, B.A. and Winkel, B.S.J. (2008) Biochemical and genetic characterization of *Arabidopsis* flavanone 3β-hydroxylase. Plant Physiol. Biochem. 46, 833–843.
[9] Saslowsky, D. and Winkel-Shirley, B. (2001) Localization of flavonoid enzymes in Arabidopsis roots. Plant J. 27, 37–48.
[10] Pelletier, M.K., Burbulis, I.E. and Shirley, B.W. (1999) Disruption of specific flavonoid genes enhances the accumulation of flavonoid enzymes and endproducts in *Arabidopsis* seedlings. Plant Mol. Biol. 40, 45–54.
[11] Kremers, G.J., Goedhart, J., van Munster, E.B. and Gadella, T.W.J. (2006) Cyan and yellow super fluorescent proteins with improved brightness, protein folding, and FRET Forster radius. Biochemistry 45, 6570–6580.
[12] Goedhart, J., Vermeer, J.E.M., Adjobo-Hermans, M.J.W., van Weeren, L. and Gadella Jr., T.W.J. (2007) Sensitive detection of p65 homodimers using red-shifted and fluorescent protein-based FRET couples. PLoS ONE 2, e1011.
[13] Karimi, M., Inzé, D. and Depicker, A. (2002) GATEWAY((TM)) vectors for *Agrobacterium*-mediated plant transformation. Trends Plant Sci. 7, 193–195.
[14] Yoo, S.D., Cho, Y.H. and Sheen, J. (2007) Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat. Protoc. 2, 1565–1572.
[15] van Munster, E.B. and Gadella, T.W.J. (2004) φFLIM: a new method to avoid aliasing in frequency-domain fluorescence lifetime imaging microscopy. J. Microsc.-Oxf. 213, 29–38.
[16] van Munster, E.B. and Gadella, T.W.J. (2004) Suppression of photobleaching-induced artifacts in frequency-domain FLIM by permutation of the recording order. Cytometry Part A 58A, 185–194.
[17] Shaner, N.C., Campbell, R.E., Steinbach, P.A., Giepmans, B.N.G., Palmer, A.E. and Tsien, R.Y. (2004) Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. Nat. Biotechnol. 22, 1567–1572.
[18] Adjobo-Hermans, M.J.W., Goedhart, J. and Gadella, T.W.J. (2006) Plant G protein heterotrimers require dual lipidation motifs of G alpha and G gamma and do not dissociate upon activation. J. Cell Sci. 119, 5087–5097.
[19] Bayle, V., Nussaume, L. and Bhat, R.A. (2008) Combination of novel green fluorescent protein mutant TSapphire and DsRed variant mOrange to set up a versatile in planta FRET-FLIM assay. Plant Physiol. 148, 51–60.
[20] Vermeer, J.E.M., van Munster, E.B., Vischer, N.O. and Gadella, T.W.J. (2004) Probing plasma membrane microdomains in cowpea protoplasts using lipidated GFP-fusion proteins and multimode FRET microscopy. J. Microsc.-Oxf. 214, 190–200.
[21] Broun, P. (2005) Transcriptional control of flavonoid biosynthesis: a complex network of conserved regulators involved in multiple aspects of differentiation in *Arabidopsis*. Curr. Opin. Plant Biol. 8, 272–279.
[22] Stracke, R., Ishihara, H., Barsch, G.H.A., Mehrtens, F., Niehaus, K. and Weisshaar, B. (2007) Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the *Arabidopsis thaliana* seedling. Plant J. 50, 660–677.
[23] Stracke, R., De Vos, R.C.H., Bartelniewoehner, L., Ishihara, H., Sagasser, M., Martens, S. and Weisshaar, B. (2009) Metabolomic and genetic analyses of flavonol synthesis in *Arabidopsis thaliana* support the *in vivo* involvement of leucoanthocyanidin dioxygenase. Planta 229, 427–445.
[24] Trabelsi, N., Petit, P., Manigand, C., d'Estaintot, B.L., Granier, T., Chaudiere, J. and Gallois, B. (2008) Structural evidence for the inhibition of grape dihydroflavonol 4-reductase by flavonols. Acta Crystallogr. Sect. D: Biol. Crystallogr. 64, 883–891.
[25] Leibundgut, M., Maier, T., Jenni, S. and Ban, N. (2008) The multienzyme architecture of eukaryotic fatty acid synthases. Curr. Opin. Struct. Biol. 18, 714–725.
[26] Mo, C.Q. and Bard, M. (2005) A systematic study of yeast sterol biosynthetic protein–protein interactions using the split-ubiquitin system. Biochim. Biophys. Acta, Mol. Cell Biol. Lipids 1737, 152–160.
[27] Turnbull, J.J., Sobey, W.J., Aplin, R.T., Hassan, A., Firmin, J.L., Schofield, C.J. and Prescott, A.G. (2000) Are anthocyanidins the immediate precursors of anthocyanidin synthase? Chem. Commun. 24, 2473–2474.
[28] Noel, J.P., Austin, M.B. and Bomati, E.K. (2005) Structure–function relationships in plant phenylpropanoid biosynthesis. Curr. Opin. Plant Biol. 8, 249–253.
[29] Stafford, H.A. (1991) Flavonoid evolution – an enzymatic approach. Plant Physiol. 96, 680–685.
[30] Dutow, P., Schmidl, S.R., Ridderbusch, M. and Stülke, J. (2010) Interactions between glycolytic enzymes of *Mycoplasma pneumoniae*. J. Mol. Microbiol. Biotechnol. 19, 134–139.
[31] Srere, P.A. (2000) Macromolecular interactions: tracing the roots. Trends Biochem. Sci. 25, 150–153.
[32] Ovádi, J. and Saks, V. (2004) On the origin of intracellular compartmentation and organized metabolic systems. Mol. Cell. Biochem. 256–257, 5–12.
[33] Winkel, B. (2004) Metabolic channeling in plants. Annu. Rev. Plant Biol. 55, 85–107.